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Diabetes-induced glomerular dysfunction: Links to a more reduced cytosolic ratio of NADH/NAD$^+$

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Diabetes-induced glomerular dysfunction: Links to a more reduced cytosolic ratio of NADH/NAD$^+$. These studies were undertaken to examine effects of elevated glucose levels on glycolysis, sorbitol pathway activity, and the cytosolic redox state of NADH/NAD$^+$ in isolated glomeruli. Blood-free glomeruli were isolated from kidneys of male, Sprague-Dawley rats using standard sieving techniques, then incubated for one hour at 37°C, pH 7.4, pO_2 ~500 torr, in Krebs bicarbonate/Hepes buffer containing 5 or 30 mM glucose. Elevated glucose levels increased glucose 6-phosphate, fructose 6-phosphate, total triose phosphates, lactate, the lactate/pyruvate ratio, sorbitol, and fructose, but did not affect *sn*-glycerol 3-phosphate, pyruvate, or *myo*-inositol levels. The more reduced glomerular cytosolic redox state (manifested by the tissue lactate/pyruvate ratio) induced by 30 mM glucose was completely abrogated by aldose reductase inhibitors added to the diet two to seven days prior to glomerular isolation. These observations, coupled with evidence linking glucose- and diabetes-induced glomerular dysfunction to increased sorbitol pathway metabolism, support the hypothesis that metabolic imbalances associated with a more reduced ratio of cytosolic NADH/NAD$^+$ (resulting from increased glucose metabolism via the sorbitol pathway) play an important role in mediating glucose- and diabetes-induced glomerular dysfunction.

Glomerular dysfunction is readily demonstrable in humans and in animals shortly after the onset of poorly controlled diabetes [1–6]. These glomerular changes, manifested as increases in blood flow, GFR, and microalbuminuria, together with associated increases in blood flow and/or vascular albumin permeation in ocular tissues, sciatic nerve, and aorta of diabetic rats, have been linked to increased metabolism of glucose via the sorbitol pathway by many [5–11] but not all [12] investigators. Increased GFR in human diabetics also is attenuated by treatment with an inhibitor of aldose reductase [13]. The precise nature of the metabolic imbalances that mediate sorbitol pathway-linked glomerular and other vascular (and neural) dysfunction, however, remains unclear.

Topical application of glucose or sorbitol to newly formed granulation tissue vessels in skin chambers increases vascular albumin permeation and blood flow in nondiabetic rats to levels observed in diabetic rats, but in the absence of insulin deficiency and associated systemic hormonal and metabolic imbalances characteristic of the diabetic milieu [6, 14]. As shown in Figure 1, these glucose-induced vascular changes are prevented by coadministration of: 1) pyruvate [15–17] which also prevents associated increases in tissue levels of 1,2-diacyl-*sn*-glycerol (DAG) in extracts of granulation tissue [15]; 2) inhibitors of aldose reductase [14] which also prevent glucose-induced increases in the ratio of lactate/pyruvate [18] (the most reliable parameter of cytosolic NADH/NAD$^+$ [19]); 3) staurosporine [15], an inhibitor of protein kinase C; and 4) *myo*-inositol [17]. The mechanism by which *myo*-inositol prevents sorbitol pathway-linked vascular dysfunction remains unclear. Taken together, these observations suggest that glucose-induced vascular dysfunction is linked to elevated tissue levels of DAG and associated activation of protein kinase C [15] resulting from a more reduced cytosolic ratio of NADH/NAD$^+$. This redox change results from increased oxidation of sorbitol to fructose which is coupled to reduction of NAD$^+$ to NADH (Fig. 1) [6, 11].

Glucose-induced increased *de novo* synthesis of DAG, increased tissue levels of DAG, and associated activation of protein kinase C also have been observed in isolated glomeruli [20, 21] and in cultured bovine retinal capillary endothelial cells [22, 23]; an increase in DAG mass has been reported in whole retina and in myocardium of diabetic rats [22, 24]; and activation of protein kinase C has been observed in bovine aortic smooth muscle cells and endothelial cells exposed to elevated glucose levels in vitro [22]. A more reduced cytosolic ratio of NADH/NAD$^+$ could promote increased *de novo* synthesis of DAG through two mechanisms (Fig. 1). First, it would favor reduction of dihydroxyacetone phosphate (DHAP) to *sn*-glycerol 3-phosphate (*sn*G3P), the first step in the pathway for *de novo* synthesis of DAG. Second, it would tend to increase the availability of substrate (DHAP) by impairing oxidation of glyceraldehyde 3-phosphate (GAP) to 1,3-bisphosphoglycerate (1,3 DPG). Evidence of a more reduced cytosolic ratio of NADH/NAD$^+$ has been observed in a variety of tissues of diabetic animals as well as in cells and tissues exposed to elevated glucose levels in vitro [6, 18, 19, 25–27].

In light of these considerations, the present studies were undertaken to examine effects on isolated glomeruli of elevated glucose levels in vitro on cytosolic NADH/NAD$^+$ reflected by the lactate/pyruvate ratio. The likelihood that acute glucose-induced in vitro metabolic imbalances of this nature may mediate increased GFR in diabetic subjects is supported by evidence that GFR is acutely increased in human subjects and in nondiabetic animals by brief intravascular infusion of glucose

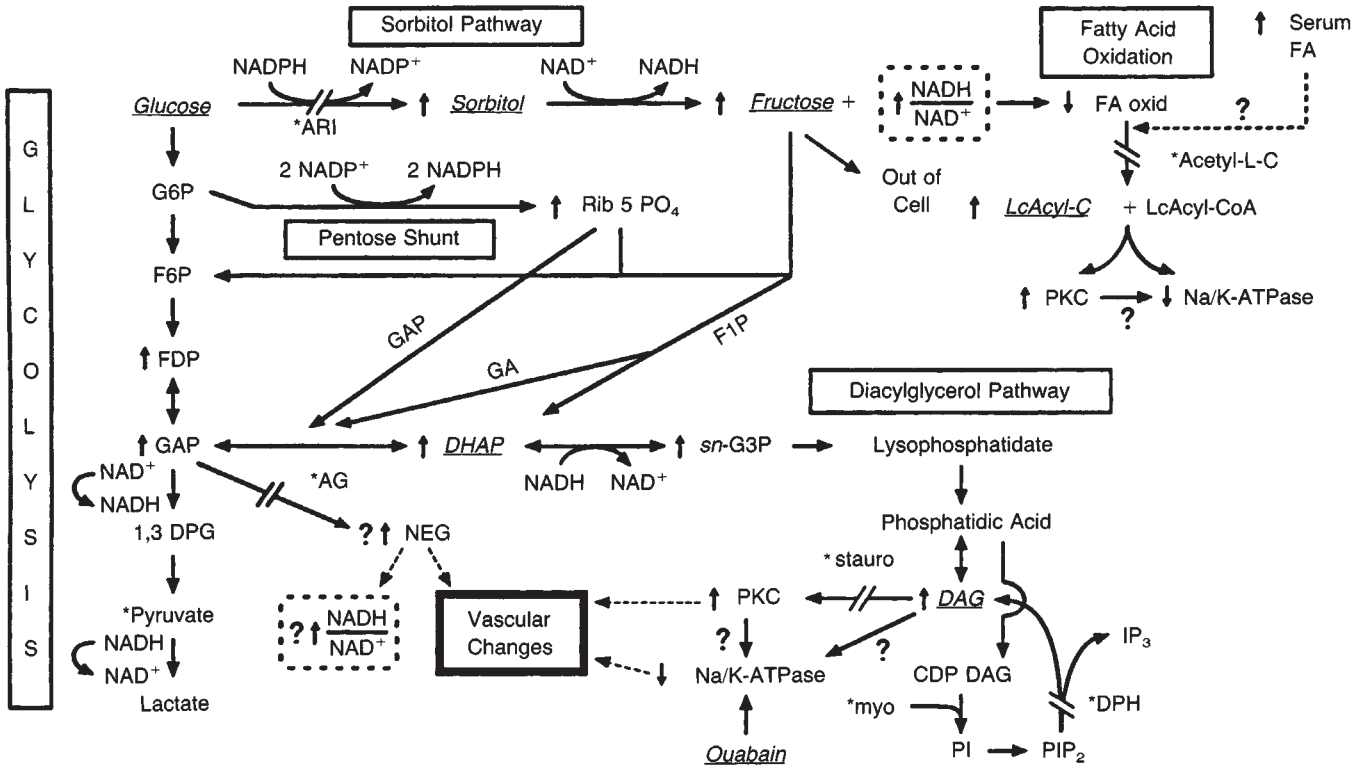

Fig. 1. *Interrelated metabolic imbalances linked to glucose- and diabetes-induced vascular dysfunction.* *Pharmacologic interventions which prevent glucose-induced vascular dysfunction are indicated by an asterisk and include: aldose reductase inhibitors (ARI), aminoguanidine (AG), staurosporine (stauro), pyruvate, *myo*-inositol (myo), diphenhydramine (DPH), and acetyl-L-carnitine (Acetyl-L-C). *sn*G3P = *sn*-glycerol 3-phosphate, FA = free fatty acid, GA = glyceraldehyde, LcAcyl-C = long-chain acylcarnitine, LcAcyl-CoA = long-chain acyl coenzyme A, NEG = nonenzymatic glycation, PKC = protein kinase C, PI = phosphatidylinositol. *Substances which mimic glucose effects on vascular function are italicized and underlined.* See references [6, 11] for further discussion of the effects of pharmacologic agents depicted in this figure.

[28, 29] or of lactate [30]. Lactate infusion would increase intra- and extracellular lactate/pyruvate ratios resulting in a more reduced cytosolic ratio of NADH/NAD^+ and hindering oxidation of NADH to NAD^+ (coupled to reduction of pyruvate to lactate).

Methods

Animal protocols

All rats used in these experiments were housed and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory Animals and in accordance with NIH guidelines on laboratory animal welfare. Rats were housed individually, allowed food and water *ad libitum*, and were on a 12-hour light/dark cycle. Glomeruli were obtained from kidneys of normal male, Sprague-Dawley rats weighing ~250 g.

Glomerular isolation protocols

For each glomerular isolation, five male Sprague-Dawley rats were anesthetized with sodium pentobarbital (35 to 45 mg/kg body weight) and anticoagulated with sodium heparin (2000 units i.p.) 10 to 15 minutes prior to surgery. A midline abdominal incision was made to expose both kidneys as well as the aorta and vena cava distal to the renal arteries. The aorta was occluded proximal to the renal arteries, a 21-gauge needle (attached to a 30 ml syringe) was inserted into the abdominal aorta below the renal arteries, a cut was made in the vena cava at the level of the renal arteries, and both kidneys were perfused by hand with ~20 ml of Krebs bicarbonate/Hepes (KH) buffer (containing in mM: NaCl, 116; KCl, 5.4; $CaCl_2 \cdot 2H_2O$, 1.8; $NaH_2PO_4 \cdot H_2O$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.8; $NaHCO_3$, 15.0; glucose, 5.0; and Hepes, 18.0), pH 7.4, 4°C. Both kidneys blanched within seconds after initiation of buffer perfusion and were moved ~20 seconds later and placed in a beaker containing KH buffer on ice. Immediately following the removal of kidneys from five rats, the capsules were stripped from all kidneys, medullary tissue was removed and discarded, and the pooled cortical tissue was minced on a ice-chilled cutting block into a fine paste with a razor blade. Glomeruli were isolated by sequential sieving at room temperature through three stainless steel sieves prewet with the KH buffer as described by Schlondorff et al [31]. The 8-inch diameter sieves were (from top to bottom) a Tyler equivalent 100 mesh (pore size 150 μm), a Tyler equivalent 170 mesh (pore size 90 μm), and a Tyler equivalent 270 mesh (pore size 53 μm). The cortical paste was gently forced through each sieve using the bottom of a 200 ml glass beaker and 2 to 3 liters of the KH buffer at room temperature for each sieve.

Glomeruli were flushed into a 50-ml conical bottom polypropylene tube using KH buffer and centrifuged for one minute at

200 rpm. The supernatant was removed and the glomeruli were resuspended in 10 ml of KH buffer containing 0.05% bovine serum albumin and 75 μM *myo*-inositol (incubation buffer). After gassing with 95% O_2 and 5% CO_2 (pO_2, ~500 torr) at 37°C, the glomeruli were allowed to rest for 15 minutes. The glomeruli were then centrifuged (2000 rpm for 30 seconds), the supernatant was removed, and the glomerular pellet was resuspended in fresh incubation buffer in a total volume of ~2.6 ml. Six hundred microliter aliquots of well-mixed glomerular suspension (20,000 to 30,000 glomeruli) were added to four 5-ml sialinized vacutainer tubes in a 37°C (shaking) water bath. Five 10-μl samples were removed for glomerular counting and to check their purity by light microscopy. The mean ratio of glomeruli to tubular fragments for all preparations was 15:1 with a standard deviation of 2.6%. After addition of substrates (in a volume of 25 μl), the tubes were gassed with humidified 95% O_2 and 5% CO_2 for five minutes and then tightly capped and incubated for one hour at 37°C.

Experimental protocols

In the first set of preliminary experiments, glomeruli were incubated in 5 or 50 mM glucose for 30, 60, or 120 minutes or in 5, 15, or 30 mM glucose for 60 minutes. Incubations were terminated by rapidly adding 60 μl of 12 N perchloric acid to the incubation buffer with shaking and the extract (tissue + medium) was saved for measurement of glycolytic intermediates. In the second series of experiments, glomeruli were incubated in 5 or 30 mM glucose ± 3 mM pyruvate or 0.07 mM tolrestat (*N*-[[5-(trifluoromethyl)-6-Methoxy-1-naphthalenyl]-thioxomethyl]-*N* methylglycine) and terminated as described above for assessment of glycolytic intermediates or as described below for quantification of glomerular polyol and *myo*-inositol content. A third set of experiments was performed on glomeruli removed from normal rats fed standard ground chow or ground chow containing tolrestat in an amount to provide 0.2 mmol/kg body weight/day or AL-1576 (spiro-(2,7-difluoro-9H-fluoren-9,4′-imidazolidine)2′,5′-dione) in an amount to provide ~0.02 mmol/kg body weight/day for two to seven days prior to glomerular isolation. In these experiments, glomeruli from control rats were incubated only in 5 or 30 mM glucose while glomeruli from rats treated with tolrestat or AL-1576 were incubated in the same buffers containing 0.07 mM tolrestat or 0.007 mM AL-1576 added in a volume of 5 μl. After a one hour incubation, glomeruli were extracted for assessment of glycolytic intermediates (tissue + medium). In a fourth experiment, glomeruli were obtained from kidneys of rats fed standard ground chow or ground chow containing tolrestat as described above, then incubated for one hour at 37°C in 2.5 ml KH buffer containing 5 or 30 mM glucose + 0.07 mM tolrestat. Glomeruli were allowed to settle to the bottom of the incubation tube during the last minute of incubation, then rapidly pipetted into a 1.5 ml microfuge tube on ice and centrifuged at 14,000 g for 30 seconds at 4°C. The remaining medium was removed and 50 μl of 12 N perchloric acid was added to the pellet. The extract (tissue only) was saved for determination of glomerular lactate and pyruvate.

Metabolic and biochemical assays

Glycolytic intermediates. Five to ten minutes following addition of perchloric acid as described above, the tubes were centrifuged, the supernatant was removed, the pH was adjusted to ~3.5, and the supernatant was assayed for fructose, *sn*-glycerol 3-phosphate, and glycolytic metabolites (glucose 6-phosphate, G6P; fructose 6-phosphate, F6P; fructose 1,6-bisphosphate, dihydroxyacetone phosphate, and glyceraldehyde 3-phosphate measured together as total triose phosphates; pyruvate, and lactate) by standard enzymatic methods [32, 33]. The pellet was saved for measurement of hydroxyproline [34] to which all the metabolic data were normalized. Prior to fructose analysis extracts were treated with glucose oxidase [35] to prevent interference with measurement of fructose by high glucose levels.

The effect of elevated glucose levels on the cytosolic ratio of NADH/NAD^+ was not measured directly, but was inferred from changes in the ratio of glomerular lactate/pyruvate; the cytosolic ratio of these metabolites is a more reliable parameter of the cytosolic ratio of free NADH/NAD^+ than measurement of the pyridine nucleotides themselves in tissue extracts [19, 36]. Since, depending on the experimental conditions and interventions performed, changes in lactate/pyruvate in the incubation medium at the end of an incubation may not correspond to the ratio of lactate/pyruvate in glomerular cytosol, experiments were performed to verify that changes in lactate/pyruvate in glomeruli plus medium (measured in the first three series of experiments) did indeed reflect the direction of changes in glomerular cytosol.

Glomerular polyol and myo-inositol. Following the one hour incubation, glomeruli were immediately centrifuged at 4°C, the pellet was resuspended in 1 ml of 0.02% sodium azide in water, internal standard was added, and the glomeruli were heated at 100°C for 15 minutes. The glomerular extracts were deproteinized with 0.3 N barium hydroxide and 0.3 N zinc sulfate, centrifuged, and sorbitol and *myo*-inositol in the supernatant were quantified (as their butyl boronate derivatives) by gas chromatography/mass spectrometry [37]. The pellet was saved for measurement of hydroxyproline content.

Statistical analysis. All data are expressed as means ± standard deviations of untransformed data. Since all experiments were performed on paired incubations, overall differences among groups for each parameter were assessed by a repeated measures analysis of variance with the SAS general linear-models procedure [38]. If the repeated measures analysis of variance test indicated that differences among groups were statistically significant at $P < 0.05$ for a given parameter, pairwise comparisons were assessed by least-square means. Because of the large variance in some parameters, a natural log transformation of the data was performed before assessment of differences between groups. The effects of aldose reductase inhibitors given in vivo on lactate/pyruvate ratios in glomeruli after incubation in 5 versus 30 mM glucose were assessed using a paired *t*-test.

Results

Effects of elevated glucose levels on glomerular glycolysis

In preliminary experiments (data not shown) glycolytic intermediates were measured in glomeruli incubated in 5 or 50 mM glucose for 30, 60, or 120 minutes. All glycolytic intermediates in the 5 mM glucose incubations reached a steady state level by 60 minutes or remained constant for 120 minutes of incubation,

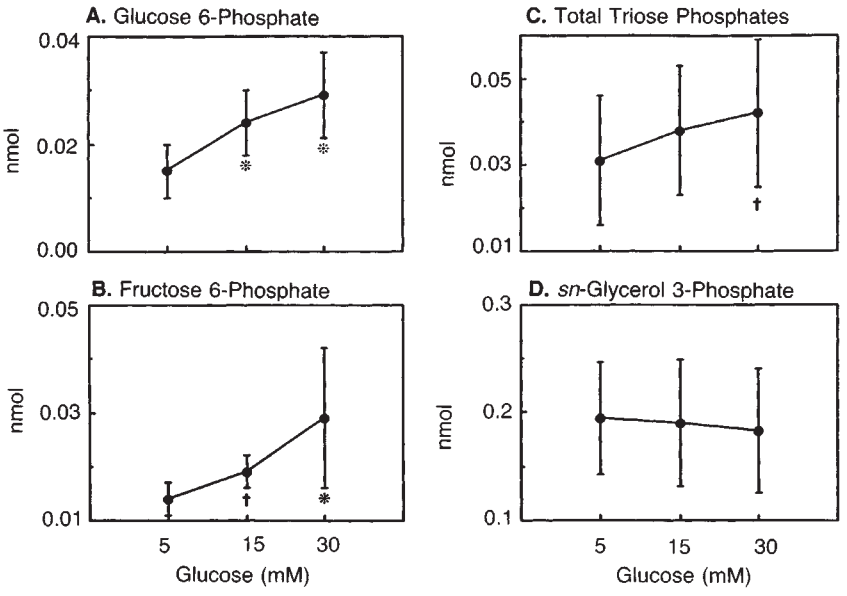

Fig. 2. *Glycolytic intermediates (glucose 6-phosphate, fructose 6-phosphate, total triose phosphates, and sn-glycerol 3-phosphate) in glomeruli incubated for one hour at 37°C in 5, 15, or 30 mM glucose.* Metabolites, expressed as nmol/μg hydroxyproline, are shown as the mean ± 1 SD ($N = 7$ for each glucose concentration). Significantly different from 5 mM glucose: $*P < 0.01$, $\dagger P < 0.03$.

with the exception of triose phosphates which increased 1.6-fold in 120 versus 30 minute incubations. Fifty millimolars of glucose increased G6P, F6P, triose phosphates, and lactate (but not pyruvate) after 30 minutes of incubation ($P \leq 0.05$). All of these metabolites remained elevated throughout 120 minutes of incubation except F6P. Pyruvate levels were significantly increased at 60 and 120 minutes ($P < 0.025$). Based on these data, subsequent incubations were carried out for 60 minutes.

Incubation of glomeruli for one hour in medium containing 5, 15, or 30 mM glucose (Figs. 2 and 3) resulted in a concentration-dependent increase in glomerular levels/production of G6P, F6P, total triose phosphates, and lactate ($P < 0.008$, 0.01, 0.02, and 0.03, respectively). Pyruvate and *sn*G3P levels remained constant between 5 and 30 mM glucose and the lactate/pyruvate ratio (Fig. 3C) increased from 11.7 ± 3.3 at 5 mM glucose to 14.8 ± 3.6 at 30 mM glucose ($P < 0.003$). Based on the results of these experiments, all subsequent incubations were performed for 60 minutes in either 5 or 30 mM glucose ± pharmacologic agents.

Effects of pyruvate and aldose reductase inhibitors added in vitro on glucose-induced metabolic changes

The effects of 30 mM glucose on glomerular levels/production of glycolytic intermediates in these experiments (Figs. 4 and 5) are consistent with those observed in the preliminary studies (Figs. 2 and 3). Sorbitol and fructose levels were increased significantly ($P < 0.001$ and $P < 0.014$, respectively) and *myo*-inositol content decreased slightly (but insignificantly) in glomeruli incubated in 30 versus 5 mM glucose (Fig. 6).

Coadministration of 3 mM pyruvate with 30 mM glucose was associated with a small increase in lactate levels (statistically insignificant vs. 30 mM glucose), an ~70% decrease in triose phosphates ($P < 0.001$ vs. 30 mM glucose) and a similar ~70% decrease in *sn*G3P ($P < 0.0002$ vs. 30 mM glucose), but had no effect on F6P or G6P (Figs. 4 and 5). Pyruvate also decreased glomerular sorbitol levels by ~60% ($P < 0.01$ vs. 30 mM glucose) but had no effect on fructose or *myo*-inositol levels (Fig. 6).

In vitro addition of 0.07 mM tolrestat to 30 mM glucose had no effect on 30 mM glucose-induced changes in lactate, pyruvate, lactate/pyruvate ratio, or triose phosphates (Figs. 4 and 5). In contrast, tolrestat significantly reduced glomerular levels of *sn*G3P (Fig. 4D). Tolrestat also largely prevented 30 mM glucose-induced increases in glomerular sorbitol levels (Fig. 6A) but had no effect on fructose or *myo*-inositol levels (Fig. 6B and C). While the 30 mM glucose-induced increase in lactate/pyruvate ratio was unaffected by the addition of 0.07 mM tolrestat in vitro (Fig. 5C), the increase was abolished in corresponding experiments with glomeruli from rats given tolrestat or AL-1576 in the diet prior to isolation of glomeruli (Fig. 7A).

Effects of elevated glucose and aldose reductase inhibitors on cytosolic redox state of glomeruli

To obtain a more reliable assessment of glomerular cytosolic $NADH/NAD^+$, lactate/pyruvate ratios were measured in glomeruli rapidly separated from incubation medium. As shown in Figure 7B, lactate/pyruvate ratios in 5 mM glucose-incubated glomeruli were almost twice those in extracts of glomeruli plus medium (Fig. 7A). Nevertheless, the ratios increased ~30% after 60 minutes of incubation in 30 mM (31.2 ± 11.6) versus 5 mM (23.8 ± 7.5) glucose, closely corresponding to the increases observed in tissue plus medium after the same duration of incubation (Fig. 7A). The lactate/pyruvate in glomeruli from rats fed ARI for seven days and incubated in 5 mM glucose plus ARI was significantly lower than that in glomeruli from control rats; 30 mM glucose-induced increase in lactate/pyruvate was prevented in 30 mM glucose-incubated glomeruli from ARI-fed rats.

Metabolite levels (in medium plus glomeruli) for glomeruli isolated from ARI-fed versus untreated rats following incubation in 5 and 30 mM glucose are summarized in Figure 8.

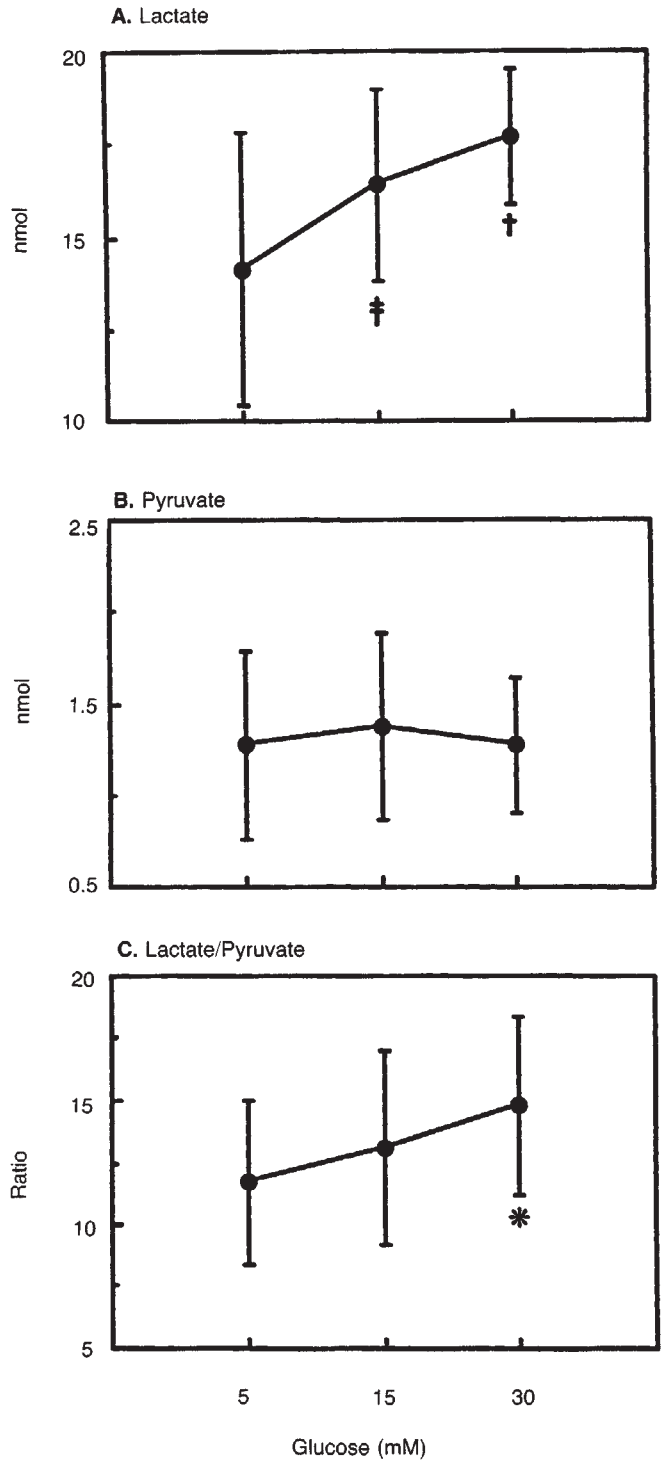

Fig. 3. *Effects of increasing glucose concentration (5, 15, and 30 mM glucose) on lactate, pyruvate, and lactate/pyruvate ratio, in glomeruli incubated for one hour at 37°C.* Metabolites, expressed as nmol/μg hydroxyproline, are shown as the mean ± 1 SD for each glucose concentration ($N = 7$). Significantly different from 5 mM glucose: $*P < 0.003$, $\dagger P < 0.03$. $\ddagger P < 0.05$.

Elevated triose phosphate levels induced by 30 mM glucose in glomeruli from untreated rats were prevented in glomeruli from ARI-fed rats (but not by ARI added in vitro only). *sn*G3P levels in 30 mM glucose-incubated glomeruli were decreased by ARI added in vitro only as well as in glomeruli isolated from ARI-fed rats. Although lactate production by glomeruli (incubated in 30 mM glucose) from ARI-fed rats tended to be lower than that by glomeruli from controls, the difference was not statistically significant.

Discussion

The demonstration that exposure of isolated glomeruli from normal rats to elevated glucose levels in vitro causes a more reduced cytosolic ratio of NADH/NAD^+ is consistent with corresponding effects of elevated glucose levels on cytosolic NADH/NAD^+ in a number of tissues from animals and in human erythrocytes, as noted in the introductory paragraphs of this paper. Furthermore, this glucose-induced glomerular redox change, like that in erythrocytes, granulation tissue, and lens, is prevented or markedly attenuated by aldose reductase inhibitors [6, 18, 25–27, 39, unpublished observations]. It should be noted that glomeruli are comprised of several different cell types (endothelial, epithelial, and mesangial cells) and the metabolic changes observed in the present studies reflect the sum of the changes in all of them. On the other hand, all of these cells are components of glomerular vessels, and the magnitude of the metabolic changes observed supports the likelihood that they reflect the metabolic state in a significant proportion of the glomerular vasculature. These observations have a number of potentially important implications for the pathogenesis of diabetic glomerulopathy.

Metabolic effects of elevated glucose levels

The ~30% increase in lactate production in glomeruli exposed to 30 versus 5 mM glucose is consistent with evidence of glucose-induced increases in lactate production in skin chamber granulation tissue [18] and increased glycolysis in bovine retinal endothelial cells [22], and increased lactate levels in retina and sciatic nerve endoneurium from diabetic rats [40]. Glucose-induced increased lactate production in all of these tissues could be explained by the presence of hexokinase(s) with a relatively high Km for glucose [41] or release (by elevated glucose levels) of hexokinase inhibition by G6P [42]. The elevated levels of G6P and F6P in 30 mM glucose-incubated glomeruli (Figs. 2 and 4) would be consistent with both possibilities.

The increase in triose phosphate levels induced by 30 mM glucose (Figs. 2, 4, and 8) is consistent with a more reduced cytosolic ration of NADH/NAD^+ since oxidation of GAP (which is in equilibrium with DHAP and FDP) to 1,3 DPG is coupled to reduction of NAD^+ to NADH (Fig. 1). Since a more reduced cytosolic ratio of NADH/NAD^+ favors *sn*G3P synthesis by reduction of DHAP (with NADH serving as the hydrogen donor), the finding that *sn*G3P levels were not elevated in 30 mM glucose-incubated glomeruli raises the possibility that utilization of *sn*G3P was increased. This interpretation is consistent with evidence that 30 mM glucose increases *de novo* synthesis of DAG in isolated glomeruli [21], since reduction of DHAP to *sn*G3P is the first step in one pathway for *de novo* synthesis of DAG (Fig. 1). These observations and interpretations also are consistent with evidence that *sn*G3P dehydrogenase activity is not high enough in some tissues (under some conditions) to maintain equilibrium between its substrates (*sn*G3P and DHAP) [19, 43].

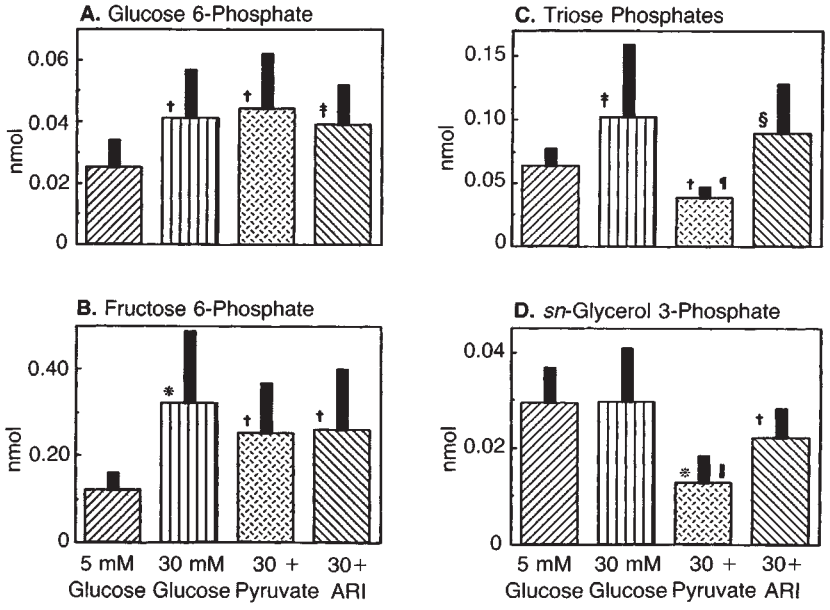

Fig. 4. *Effects of 3 mM pyruvate and 0.07 mM tolrestat on glycolytic intermediates (expressed as nmol/μg hydroxyproline, N = 9 for each group, mean ± 1 SD) in glomeruli incubated with 30 mM glucose for one hour at 37°C (N = 9 for each group).* Significantly different from 5 mM glucose: $*P \leq 0.001$, $\dagger P \leq 0.005$, $\ddagger P \leq 0.001$, $\S P < 0.05$. Significantly different from 30 mM glucose: $\| P \leq 0.0002$, $\P P < 0.006$.

The increased sorbitol levels in 30 mM glucose-incubated glomeruli (Fig. 6) is consistent with previous reports [12, 44] and, together with the increased fructose levels (Fig. 6) and the prevention of 30 mM glucose-induced increase in the lactate/pyruvate ratio by aldose reductase inhibitors, supports the hypothesis that increased oxidation of sorbitol to fructose contributes significantly to the more reduced cytosolic ratio of $NADH/NAD^+$ in glomeruli exposed to elevated glucose levels.

Effects of aldose reductase inhibitors and pyruvate on glucose-induced metabolic imbalances

Glucose-induced increases in lactate/pyruvate ratios (in glomeruli plus medium) were relatively unaffected by coadministration of ARI in vitro only (Fig. 5), but were prevented in glomeruli from ARI-fed rats (Fig. 7A). These discordant findings may be explained by a relatively slow rate of penetration of glomerular cells by ARI. By the time inhibitory levels were attained in glomeruli exposed to ARI only in vitro, it was too late to normalize the lactate/pyruvate ratio in the medium (most of the lactate and pyruvate are recovered in the medium rather than in the cells). In contrast, excess intracellular sorbitol formed before inhibition of aldose reductase activity was achieved would still be readily oxidized to fructose, that is, "normalized," during the latter part of the incubation. This interpretation is consistent with evidence that sorbitol levels in red cells and other tissues decrease rapidly following normalization of glucose levels [45–47]. The observation that tolrestat decreased tissue lactate/pyruvate ratio in glomeruli incubated in 5 mM glucose suggests that flux of glucose via the sorbitol pathway impacts significantly on glomerular cytosolic $NADH/NAD^+$ even at physiological glucose levels.

Aldose reductase utilizes NADPH as the hydrogen donor for the reduction of glucose to sorbitol. This reaction results in a more oxidized ratio of $NADP^+/NADPH$ (Fig. 1). In contrast, sorbitol dehydrogenase utilizes NAD^+ as the hydrogen acceptor for oxidation of sorbitol to fructose. This reaction results in a more reduced cytosolic ratio of $NADH/NAD^+$ as noted earlier. Therefore, the 30 mM glucose-induced increases in lactate/pyruvate (Fig. 7) and their prevention by ARI appear to be more closely linked to redox changes resulting from increased oxidation of sorbitol to fructose than to reduction of glucose to sorbitol. This interpretation is supported by several lines of evidence. First, although (as noted earlier) the ratio of pyridine nucleotides in cellular extracts may not accurately reflect the cytosolic ratio of free $NADH/NAD^+$, it is noteworthy that 30 mM glucose-induced changes in $NADH/NAD^+$ in glomerular extracts are indeed consistent with changes in lactate/pyruvate ratio in the incubation medium (data not shown). Second, topical application of sorbitol to granulation tissue vessels in vivo causes vascular dysfunction, like that induced by glucose, which is prevented by coadministration of pyruvate but not by aldose reductase inhibitors [16, unpublished observations]. Third, the ratio of lactate/pyruvate and the levels of other metabolites (triose phosphates and *sn*G3P) modulated by $NADH/NAD^+$ in human erythrocytes are markedly increased during incubation with sorbitol or xylitol (xylitol also is a substrate for sorbitol dehydrogenase) [39, 48], and these increases are prevented by pyruvate [39]. The decrease in *sn*G3P levels (and the trend for triose phosphates to decrease, albeit not statistically significant) in 30 mM glucose-incubated glomeruli from ARI-fed rats (Fig. 8) is consistent with the associated normalization of the cytosolic lactate/pyruvate ratio (Fig. 7B). The observation that *sn*G3P levels in 30 mM glucose-incubated glomeruli from ARI-fed rats were lower than those in 5 and 30 mM glucose-incubated glomeruli from control rats (Fig. 8) may reflect greater inhibition (by ARI) of synthesis than utilization of *sn*G3P (due to differences in oxidation/reduction potentials and equilibrium constants of *sn*G3P dehydrogenase and the enzymes that utilize *sn*G3P for lipid synthesis) [19, 43].

The pyruvate-induced decrease in triose phosphates, *sn*G3P, and sorbitol (Figs. 4 and 6) are consistent with corresponding pyruvate effects on glucose-induced changes in cytosolic $NADH/NAD^+$ and on $NADH/NAD^+$ modulated metabolite levels (including sorbitol and fructose) in human red cells and in skin chamber granulation tissue [18, 25, 39, unpublished observations]. All of these effects of pyruvate are consistent with

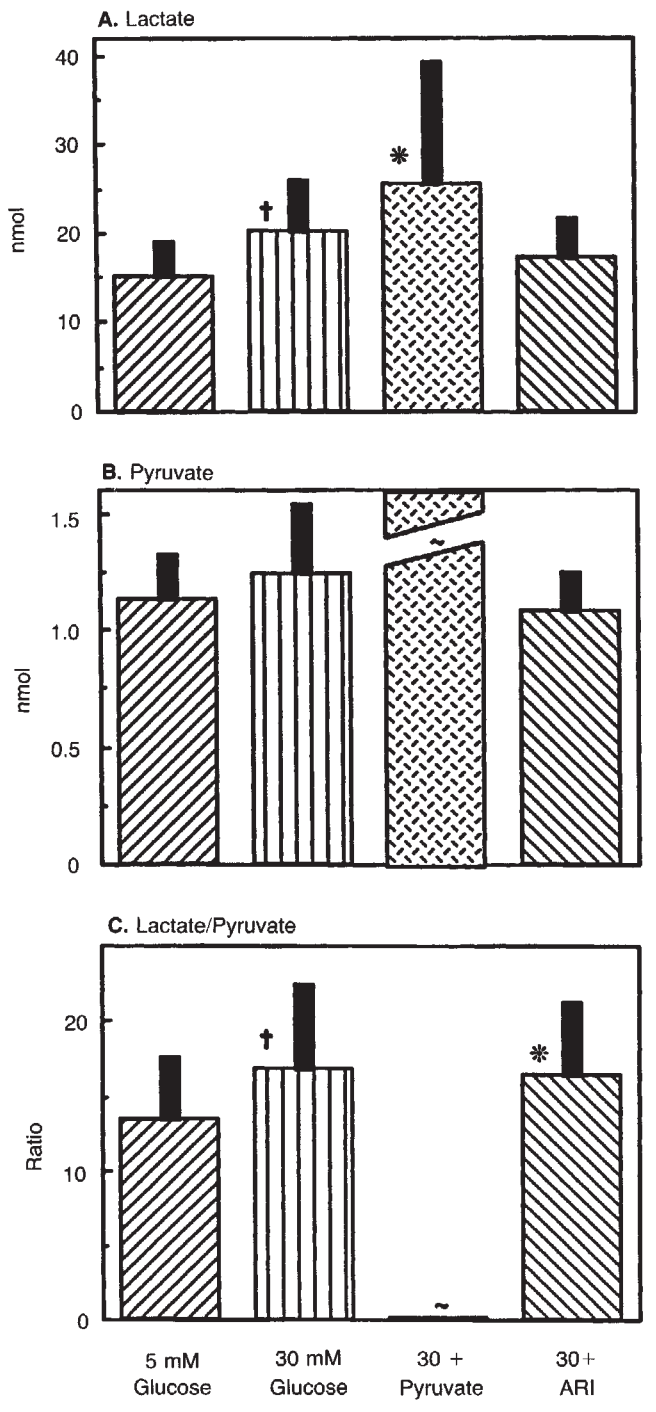

Fig. 5. *Effects of 3 mM pyruvate and 0.07 mM tolrestat on lactate, pyruvate, and lactate/pyruvate ratio in glomeruli incubated with 30 mM glucose for one hour at 37°C.* Metabolites, expressed as nmol/μg hydroxyproline, are shown for each incubation as the mean ± 1 SD ($N = 9$ for each group). The amount depicted for pyruvate (**B**) in the 30 mM glucose + 3 mM pyruvate group is an approximate value (indicated by the ~) derived by dividing lactate produced by glomeruli by pyruvate added to the medium. It is shown merely to indicate that addition of 3 mM pyruvate to the medium results in higher pyruvate levels than those produced by glomerular metabolism. Significantly different from 5 mM glucose: $^*P < 0.001$, $\dagger P \leq 0.005$.

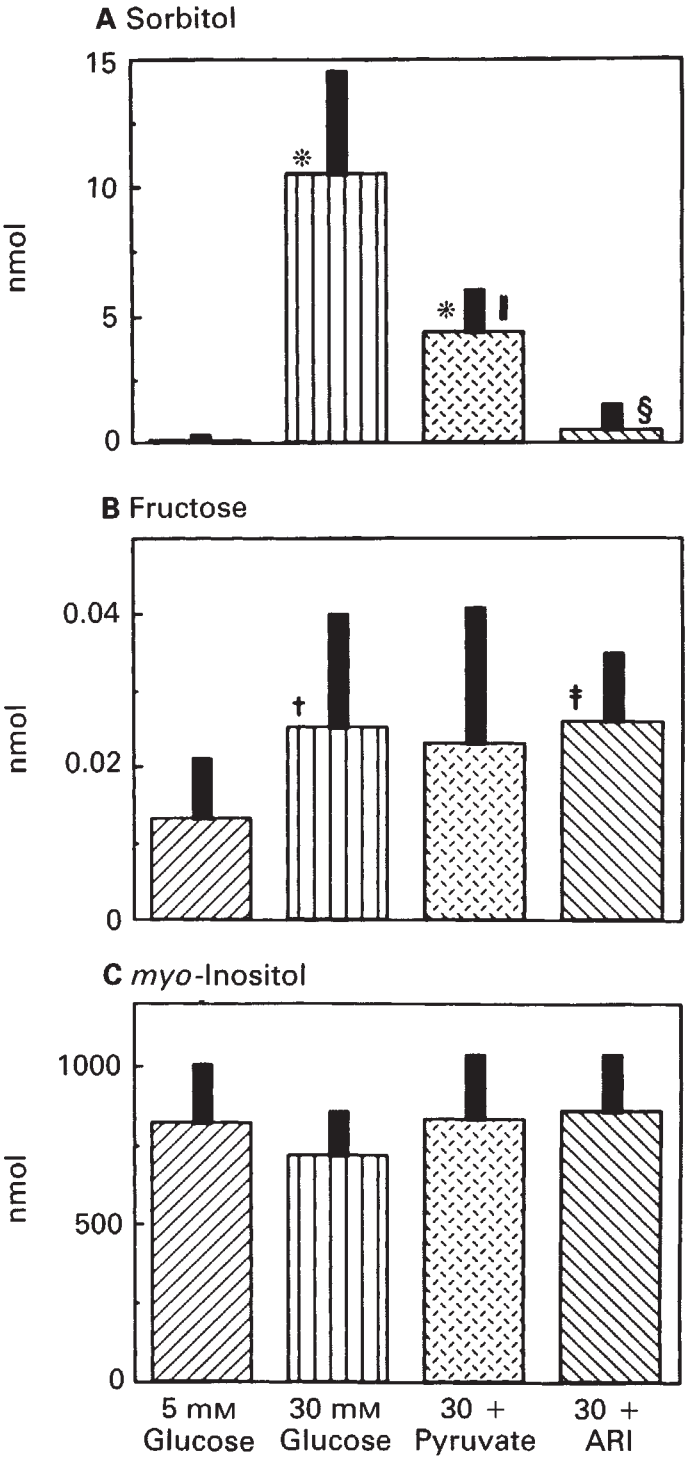

Fig. 6. *Effects of 3 mM pyruvate and 0.07 mM tolrestat on sorbitol, fructose, and myo-inositol accumulation in glomeruli obtained from normal rats after incubation in 30 mM glucose for one hour at 37°C ($N = 6$).* Results are expressed as nmol/μg hydroxyproline and are mean ± 1 SD. Significantly different from 5 mM glucose: $^*P \leq 0.001$, $\dagger P < 0.014$, $\ddagger P < 0.04$. Significantly different from 30 mM glucose: $\S P < 0.014$, $\| P < 0.011$.

oxidation of NADH to NAD^+ by pyruvate at a rate faster than NAD^+ is reduced to NADH by oxidation of sorbitol to fructose (Fig. 1). The apparent failure of pyruvate to increase fructose

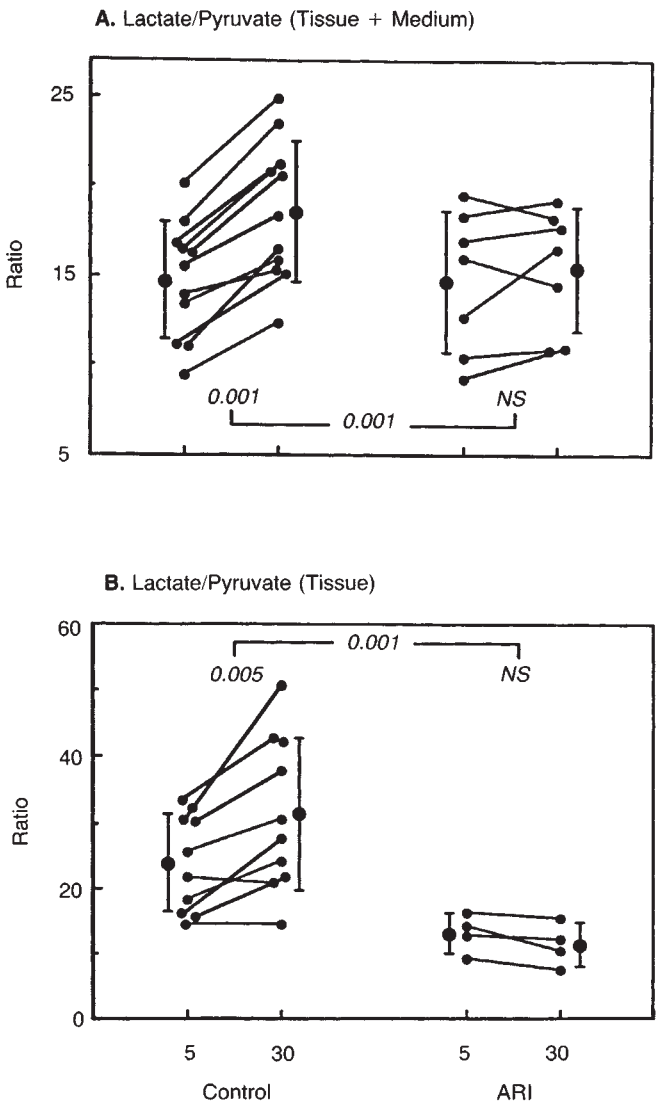

Fig. 7. *Effects of 5 mM and 30 mM glucose ± tolrestat on the glomerular tissue lactate/pyruvate ratio* (**A**) *or on tissue plus medium lactate/pyruvate ratio* (**B**). Glomeruli used for incubation in 5 or 30 mM glucose + tolrestat were obtained from nondiabetic rats fed tolrestat in their diet (0.02 mmol/kg body weight) for 7 days prior to glomerular isolation. Since these are paired observations, the lactate/pyruvate ratios from each incubation are connected by lines. Mean ± 1 SD is also indicated.

levels in 30 mM glucose-incubated glomeruli, despite a marked reduction in sorbitol levels, may be due to the presence of fructokinase in the glomeruli and/or in the small number of contaminating tubules, portions of which contain large amounts of the enzyme [49].

These observations raise several important related questions regarding: 1) the reliability of measurements of tissue sorbitol levels alone (without fructose measurements) as an index of sorbitol pathway activity, 2) how much of an increase in sorbitol pathway activity can be tolerated by cells/tissues without pathophysiological consequences, and 3) how readily reversible increases in sorbitol pathway activity contribute to poorly reversible glucose- and diabetes-induced metabolic imbalances, as well as functional and structural angiopathy (including glomerulopathy) and neuropathy. Tissue sorbitol levels are determined not only by intracellular glucose levels, but also by the ratio of sorbitol dehydrogenase to aldo-keto reductase enzyme activities [50]. Thus, if the ratio of sorbitol dehydrogenase to aldose reductase in a tissue is high, elevated tissue glucose levels can markedly increase glucose metabolism via the sorbitol pathway to fructose (Fig. 1), and induce sorbitol pathway-mediated cellular injury, with little or no increase in tissue sorbitol content.

The level to which sorbitol pathway activity can be increased without causing metabolic and pathophysiological consequences remains unclear and may vary considerably in different tissues. It appears likely that relatively small increases in sorbitol pathway activity may be sufficient to cause vascular dysfunction in many tissues. This view is supported by the observation that in diabetic rats treated with two different doses of an ARI (AL-1576), both of which normalized sorbitol levels in every tissue examined, only the higher dose decreased microalbuminuria and normalized GFR and vascular albumin permeation in ocular tissues, sciatic nerve, and granulation tissue [5]. These observations have important implications regarding the interpretation of experiments in which ARI have been reported to normalize sorbitol levels but fail to prevent associated glucose-induced changes in protein kinase C activity [20, 23] and diabetes-induced increases in GFR [12] and glomerular structural changes [12]. Differential effects of ARI on metabolic imbalances, functional, and/or structural changes in the same cells and tissues [23, 51–53] may reflect the differential impact of partial normalization of cytosolic NADH/NAD^+ on dehydrogenase enzymes with different oxidation/reduction potentials and equilibrium constants (and on the relative concentrations of their reduced and oxidized substrates).

The nature of the mechanism(s) by which transient increases in sorbitol pathway activity may induce poorly reversible functional and structural glomerular changes (and other vascular and neural complications of diabetes) also remains unclear. In view of evidence linking increased tissue DAG levels (and associated increased protein kinase C activity) to increased sorbitol pathway activity in granulation tissue [15], it can be postulated that transient elevations in DAG may cause much longer lasting effects through activation and membrane association of protein kinase C and phosphorylation of protein kinase C substrates.

Implications of a more reduced cytosolic NADH/NAD^+ ratio for the pathogenesis of diabetic nephropathy

One mechanism by which increased sorbitol pathway activity may induce poorly reversible vascular changes is by production of metabolites capable of nonenzymatically glycating intracellular constituents such as triose phosphates (which are among the most reactive naturally occurring glycating agents in tissues [54]) and fructose 3-phosphate and 3-deoxyglucosone [55]. The likelihood that nonenzymatic glycation may contribute to glucose- and diabetes-induced vascular dysfunction is supported by evidence that aminoguanidine (a nucleophilic hydrazine compound believed to prevent formation of advanced glycation end-products [56]) prevents sorbitol-, glucose-, and diabetes-induced increases in vascular albumin permeation [57–59].

A more reduced cytosolic ratio of NADH/NAD^+ could impact on at least two important pathways of lipid metabolism

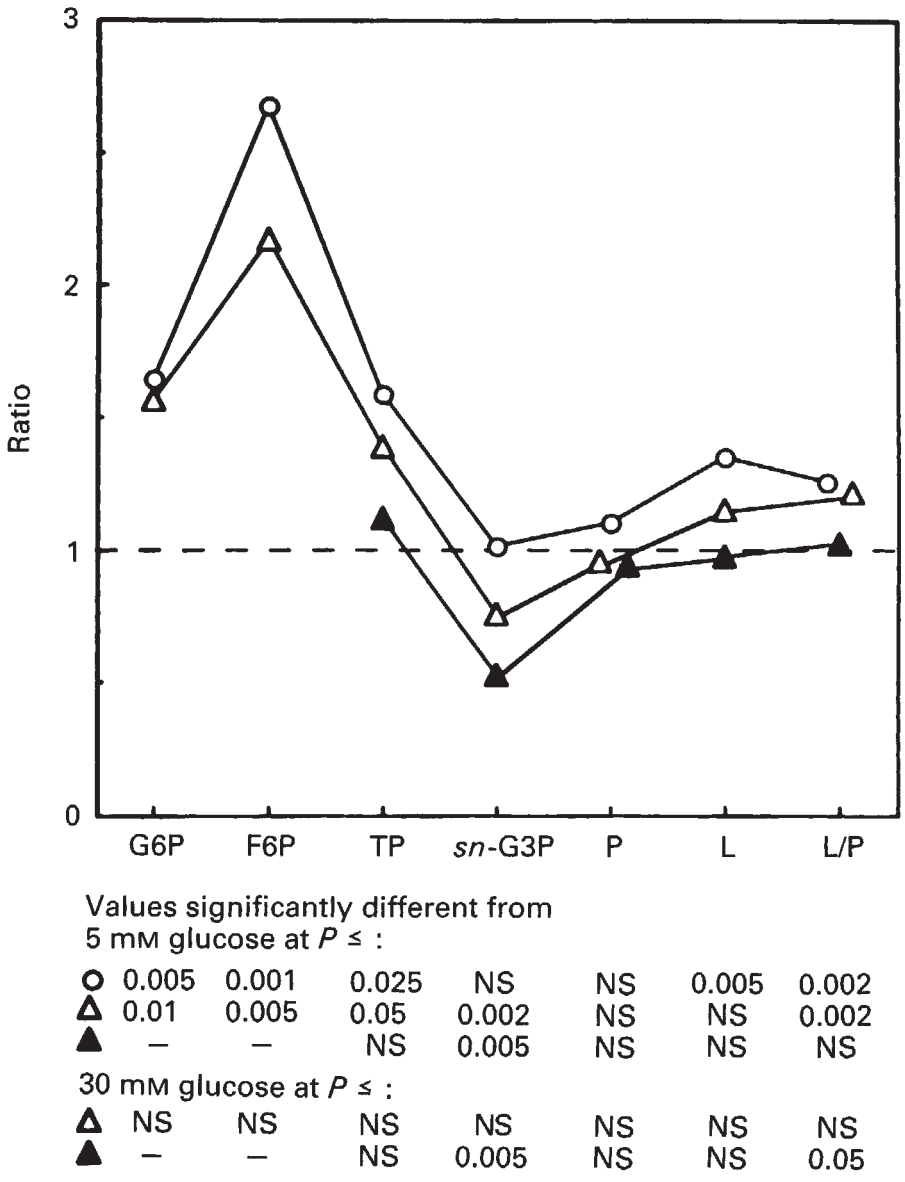

Fig. 8. *Glycolytic metabolite levels after incubation in 5 and 30 mM glucose of glomeruli from rats fed ARI in the diet (**Methods**) prior to glomerular isolation versus metabolite levels in glomeruli isolated from untreated rats with ARI added in vitro only.* Metabolite levels are expressed as a ratio of the corresponding level in 5 mM glucose-incubated glomeruli. The dashed line (ratio = 1) represents the metabolite level in 5 mM glucose-incubated glomeruli. Open circles = 30 mM glucose; open triangles = 30 mM glucose + 0.07 mM ARI added to incubation medium in vitro; closed triangles = 30 mM glucose + ARI (tolrestat or AL-1576) added to the diet and also added to the incubation medium (**Methods**). G6P = glucose 6-phosphate, F6P = fructose 6-phosphate, TP = total triose phosphates (fructose 1,6-bisphosphate, dihydroxyacetone phosphate, and glyceraldehyde 3-phosphate), *sn*G3P = *sn*-glycerol 3-phosphate, P = pyruvate, L = lactate, and L/P = lactate/pyruvate ratio. G6P and F6P data were not available for the group given ARI in the diet (represented by closed triangles).

resulting in: 1) increased *de novo* synthesis of DAG, and 2) impaired β oxidation of long chain fatty acyl CoA in mitochondria. As discussed above, a more reduced cytosolic ratio of NADH/NAD^+ would favor glucose-induced increased *de novo* synthesis of DAG and associated activation of protein kinase C (Fig. 1) observed in isolated glomeruli exposed to elevated glucose levels in vitro [20, 21]. The putative importance of this cascade of metabolic imbalances is consistent with amelioration by *myo*-inositol of vascular dysfunction (including increases in GFR and in urinary excretion of IgG) in diabetic rats [8, 60], since elevation of tissue *myo*-inositol levels would favor incorporation of DAG into phosphatidylinositol (Fig. 1), thereby lowering DAG levels and normalizing impaired phosphatidylinositol metabolism [61, 62]. This interpretation is consistent with evidence that agonist-induced increases in DAG and CDP-DAG (associated with breakdown of PIP_2) in a variety of tissues are prevented and/or reversed by *myo*-inositol [63–66]. In addition, excess cytosolic reducing equivalents (NADH) generated by increased oxidation of sorbitol to fructose can be transported into the mitochondria (by the malate-aspartate shuttle). In ischemic myocardium, an increase in mitosolic NADH/NAD^+ inhibits mitochondrial β oxidation of long-chain fatty acyl CoA (Fig. 1) [11], which accumulate as long-chain acyl esters of CoA and carnitine [67]. These long-chain acyl esters, like DAG, are potent modulators of the activity of several enzymes (including protein kinase C, Na^+/K^+-ATPase, and Ca^{++}-ATPase) implicated in the pathogenesis of diabetic complications. While the role of this putative cascade of metabolic imbalances in the pathogenesis of diabetic glomerulopathy remains unknown, metabolic imbalances of this nature could explain the apparent links between elevated plasma lipid levels, microalbuminuria, and nephropathy in diabetic humans and animals [68–71].

In conclusion, these studies demonstrate that elevated glucose levels lead to a more reduced ratio of cytosolic NADH/NAD^+ in glomeruli which is linked to increased flux of glucose

via the sorbitol pathway. They also suggest several candidate mechanisms by which a more reduced glomerular cytosolic ratio of NADH/NAD^+ may modulate lipid metabolism and nonenzymatic glycation and contribute to the pathogenesis of diabetic nephropathy. While these observations implicate increased sorbitol pathway metabolism in the pathogenesis of glomerular dysfunction in diabetic animals, further studies are needed to elucidate the role of sorbitol pathway metabolism in the pathogenesis of diabetic glomerulopathy in human subjects.

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Appendix. List of abbreviations

ARI	aldose reductase inhibitor
CDP-DAG	cytidine diphosphate-DAG
DAG	1,2-diacyl-*sn*-glycerol
DHAP	dihydroxyacetone phosphate
1,3 DPG	1,3-bisphosphoglycerate
FDP	fructose 1,6-bisphosphate
F6P	fructose 6-phosphate
GAP	glyceraldehyde 3-phosphate
G6P	glucose 6-phosphate
KH	Krebs-HEPES buffer
L	lactate
L/P	lactate/pyruvate ratio
P	pyruvate
PIP_2	phosphatidylinositol-bisphosphate
*sn*G3P	*sn*-glycerol 3-phosphate
TP	total triose phosphates

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